Optimization of Biotin Enrichment Protocols for the Analysis of Biotinylated Proteins by LC-MS/MS

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Abstract

The strong interaction between biotin and avidin/streptavidin serves as the basis for using biotin as a tag for enrichment of a targeted population of proteins from highly complex protein mixtures and purification of biotin tagged-proteins using avidin affinity chromatography. However, the recovery of biotinylated proteins from the streptavidin-based materials and discrimination between the background contamination and the subpopulation of interest remain major challenges. In this study, we first determined a cut-off ratio for detecting the true positive biotinylated proteins. Next, using a SILAC labelled HEK cell line stable transfected with promiscuous biotin ligase (BirA*), we performed four different strategies used widely for recovery of biotinylated proteins: on bead digest using two sets of washing buffers, digestion prior to incubation with beads, and elution from beads and in-gel digest. We then compared the results in terms of the number of identified proteins, reproducibility, and enrichment level. Our results demonstrate that the highest number of identification and biotinylation is reached by on bead digestion followed by off gel fractionation (method 1).

Introduction

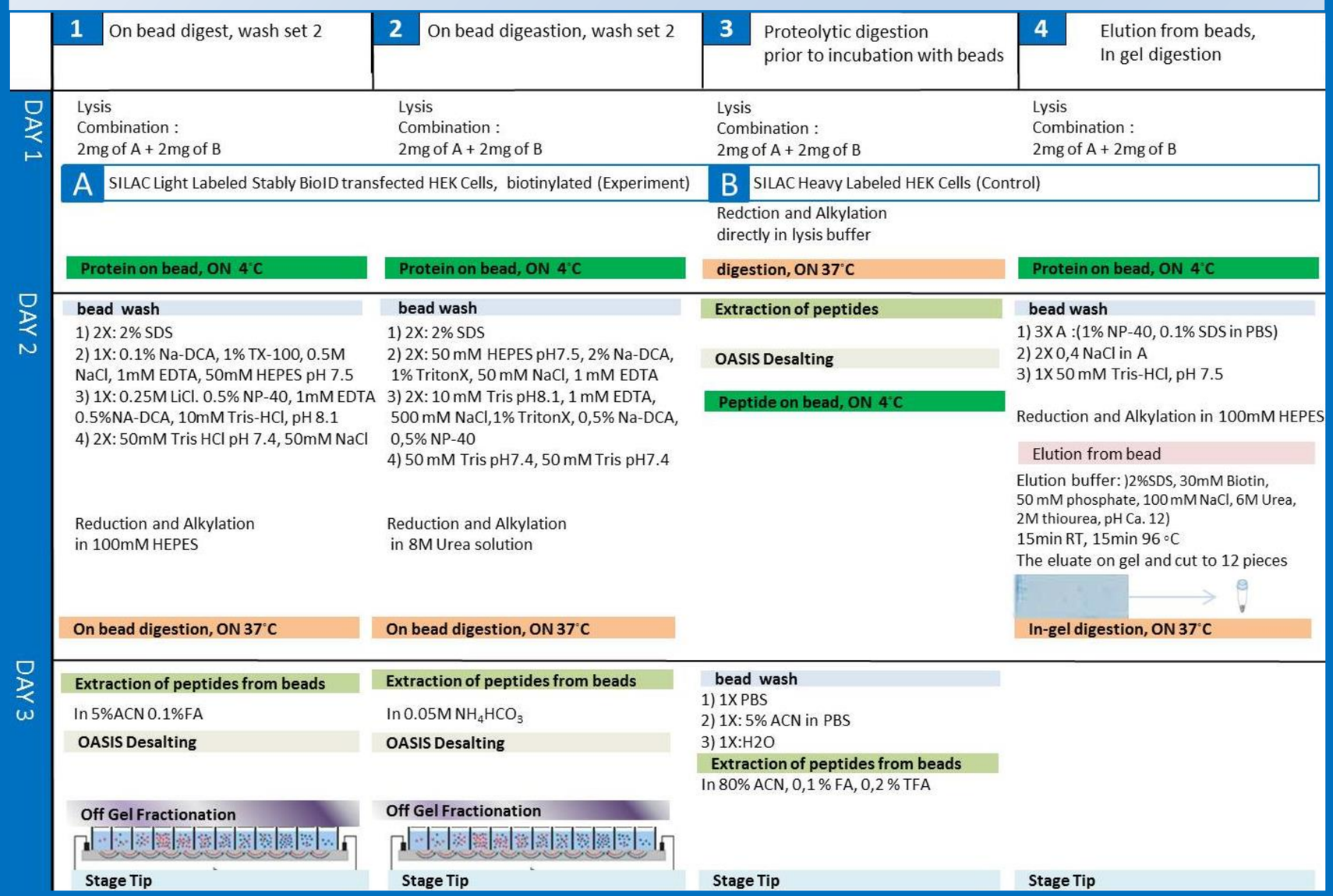
The extraordinary strong interaction between biotin and avidin has been exploited for more than 50 years in numerous purification and detection strategies[1]. In proteomics, avidin/streptavidin-biotin interaction is primarily used in interactome studies for enrichment of a subpopulation of proteins for subsequent Mass Spectrometry (MS) analysis. One of the popular approaches taking advantage of the specific biotin-avidin interaction for screening of interacting proteins in native cellular environments is the BirA* approach[2]. BirA* is a mutant form of BirA (R118G), a 35-kD DNA-binding biotin protein ligase in *Escherichia coli,* which enables nonspecific biotinylation of the proteins in a proximity-dependent fashion in a radius of 10 nM in cells. In cells expressing BirA*, upon supplementation of the culture with biotin, BirA* starts adding biotin to vicinal proteins. The biotin-tagged population of proteins should then be selectively isolated using avidin containing materials and processed to MS analysis for identification.

Albeit the extraordinary stability of the avidin-biotin bond (10^{15} M^{-1}) results in very efficient capture of biotinylated proteins, the recovery of biotinylated proteins from avidin based reagents is difficult and remains a major challenge. An effective purification method should allow effective recovery of all biotinylated proteins and enable discrimination between biotinylated and background proteins. Different methods for affinity capture of biotinlytaed proteins using streptavidin supports have been used[3,4,5]; in this study we compared four of these strategies using streptavidin beads.

Methods

Human Embryonic Kidney (HEK) 293 cells stable transfected with BirA* were generated and labelled with light SILAC amino acids (Lys-0 and Arg-0); cells were incubated with 50µM biotin for 24 hours and harvested. Heavy SILAC labelled HEK (Lys-8 and Arg-10) were used as control. Equal amounts of cell lysate from experiment and control cells were mixed and incubated with streptavidin beads for affinity capture of biotinylated proteins. Recovery of biotinylated proteins was carried out using four different methods, each in three replicates; 1 and 2: On bead digestion of proteins and off-gel fractionation using two different sets of washing buffers; 3. Digestion of proteins prior to on bead biotin capture; and 4. Elution from the bead followed by in-gel digestion. In addition, in order to distinguish between unspecific (non-biotinylated) and specific (biotinylated) identified proteins and to determine a cut-off for the acceptance of true positive biotinylation, HEK 293 were labelled with Light (Lys-0 and Arg-0), medium (Lys-4 and Arg-6), and heavy (Lys-8, Arg-10) SILAC amino acids and lysed. Proteins were combined and enriched using streptavidin affinity beads; all samples were analysed by LC-MS/MS.

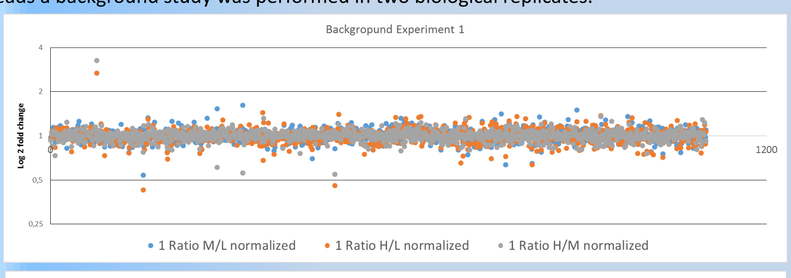
Detailed Workflow of the Affinity Enrichment Methods

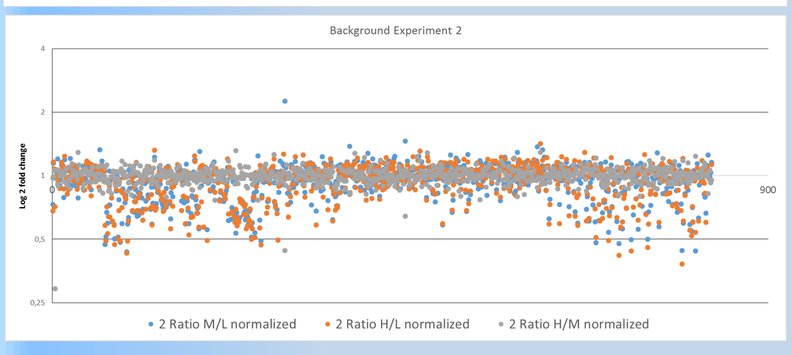


Results

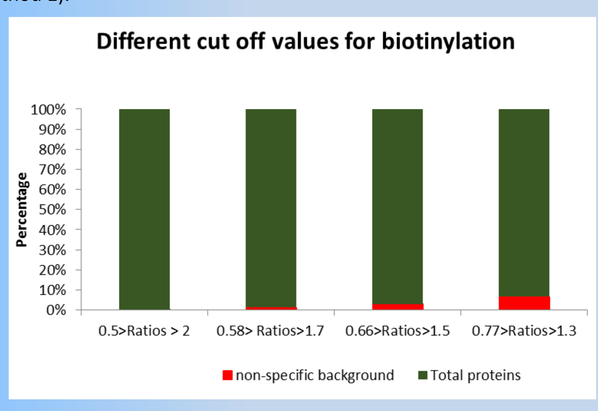
Background Study (Determination of cut-off for Biotinylation)

In order to identify the non-biotinylated proteins which unspecifically bind to streptavidin beads a background study was performed in two biological replicates.





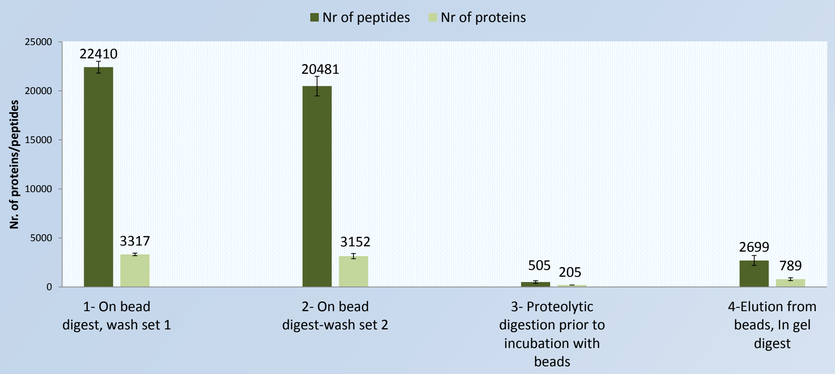
The graphs show scatter plots of log2 fold change values for the two background experiments performed for determination of a cut-off for the acceptance of true positive biotinylation. HEK 293 cells (that were not treated with biotin) were labelled with light, medium, and heavy SILAC labels, mixed and processed by the on bead digest protocol using the first set of wash buffers (method 1).



Different cut-offs and their inverse values were evaluated to distinguish between unspecific (non-biotinylated) and specific (biotinylated) proteins. In average only 0.6 % of the identified proteins in these background studies had SILAC ratios above the cut off 2 or below the inverse of it (that is 0.5). 1.23 % of proteins had SILAC ratios outside of the range of 0.58 - 1.7. 2.93 % of identified proteins had ratios below 0.66 or above 1.5; and 6.5% possessed ratios were less than 0.77 or greater than 1.3. To ensure assigning biotinylation only to true positive proteins in experiment studies, we set the cut off to the SILAC ratio 2; accordingly, proteins with experiment/control ratios ≥ 2 are considered biotinylated.

Identification Number in each Method

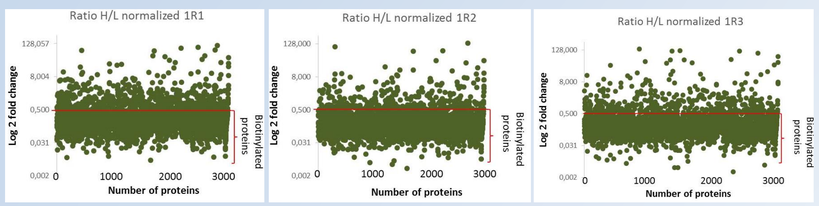
The bar graphs show the number of identified proteins and peptides in each method. Data shown are average and error bars represent ±S.D. On bead digestion using the first set of wash buffers (method 1) resulted in identification of 22410 peptides on average, and 3487, 3148, and 3325 proteins (2438 common). On bead digest with second composition of wash buffers (method 2) resulted in identification of 20481 peptides on average, 3012, 2924, and 3519 proteins (2159 common). proteolytic digestion followed by incubation of peptides on bead (method 3) yielded in finding 505 peptides on average, and 128, 243, and 224 proteins (85 common). Elution of biotinylated proteins followed by in gel digestion (method 4) resulted in 2699 peptides on average, and 597, 820, and 949 proteins (398 common).



The highest number of identification and maximum number of common proteins were obtained using on bead digest method processed with the first set of wash buffers.

Number of Biotinylated Proteins

Based on the results of the background study, if the SILAC experiment/control ratio exceeds 2 (the cut-off calculated for biotinylation), the respective protein is considered to be biotinylated in the experiment population.

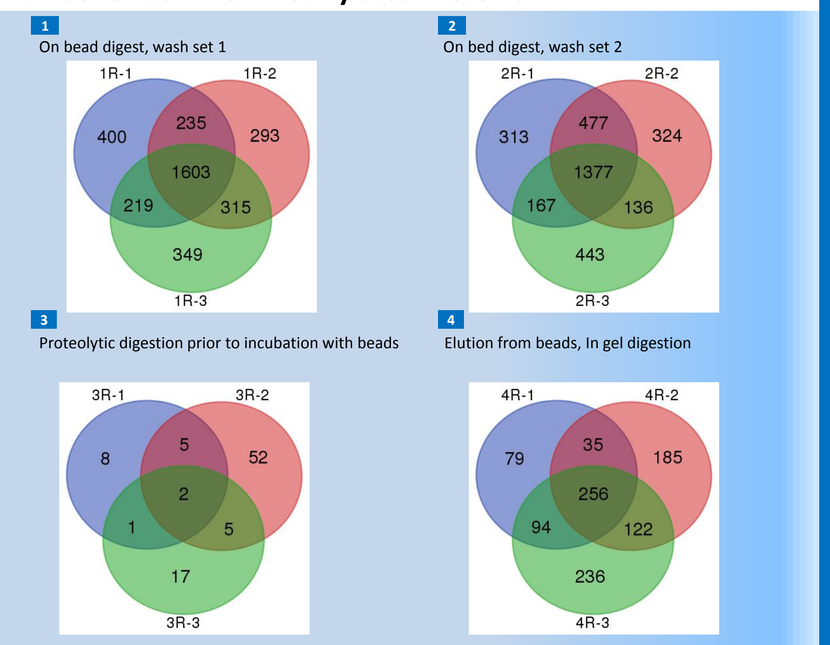


The graph show the scatter plots of the log2 fold change values for BirA* experiments using SILAC heavy labelled HEK 293 cells as control and light labelled BirA* stable transfected HEK 293 cells as experiment, processed by on bead digestion using first set of wash buffers (method 1). Based on the cut-off determined in background studies, 71.5, 78.6, and 75.7 % (in average 75%) of the proteins in replicate 1,2, and 3, respectively, have SILAC H/L ratios less than 0.5 and therefore are considered biotinylated.

Biotinylation Levels in each Method

The graph shows the enrichment level (percentage of biotinylated proteins) in each of the three replicates of four methods. The on bead digestion using the first set of wash buffers (method 1) resulted in identification of on average 75 % biotinylated proteins. The on bead digest using the second composition of washing buffers (method 2) resulted in detection of 72% biotinylated proteins on average. Digestion prior to incubation of peptides on bead (method 3) yielded in averaged 17% enrichment for biotinylated proteins. Elution of biotinylated proteins followed by in gel digestion (method 4) resulted in detection of biotinylation in 75% of the proteins.

Number of Common Biotinylated Proteins



The venn diagrams show the number of common biotinylated proteins among the 3 replicates of each method. 64 % of the population of the biotinylated proteins in the on bead digest method 1 are found in all three replicates. The percentages of common proteins are 60%, 6%, and 43% for the on bead digest method 2, proteolytic digestion prior to incubation with beads, and elution from beads followed by in gel methods, respectively.

Conclusion

Using the background experiments we were able to determine a cut-off to distinguish between background proteins and biotinylated proteins. We compared different ranges and assigned experiment/ control ratios ≥ 2, to biotinylated proteins. Smaller cut off ratios resulted in higher identification numbers; however, they increase the chance of introducing proteins binding nonspecifically to streptavidin beads to the list of biotinylated proteins. Based on the cut-off set in the background study, we compared the results of methods for the affinity enrichment of biotinylated proteins. Except for the digestion of proteins prior to on bead biotin capture method, the other methods resulted in similar enrichment capacity (70-75%) for biotinylated proteins. On bead digest using the first set of wash buffers however, resulted in highest number of identified proteins (3317, on average) and highest percentage of common biotinylated proteins among replicates (64% of biotinylated proteins were common among replicates), suggesting it to be the method of choice for affinity capture of biotinylated proteins.

References

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